Bacteria Associated with the Guts of Two Wood-Boring Beetles: *Anoplophora glabripennis* and *Saperda vestita* (Cerambycidae)

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ABSTRACT Commensal microorganisms have significant impacts on the health of many insect hosts. Little is known, however, about the structure of commensal bacterial communities associated with the Cerambycidae, despite the important roles this large family of herbivorous endophytic insects plays in ecosystem processes, economic losses to ornamental and forest trees, and biological invasions. We analyzed the bacterial commensal communities of the exotic Asian longhorned beetle, *Anoplophora glabripennis*, and the native linden borer, *Saperda vestita*, by randomly sequencing 16S rRNA gene fragments from bacterial DNA extracted directly from the gut of larvae. The 16S rRNA gene sequences sampled from *S. vestita* were derived entirely from the γ-Proteobacteria phylum of Bacteria. In contrast, the gut of *A. glabripennis* larvae contained members of the α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. A better understanding of insect-microbe interactions may lead to new strategies to reduce the effects of these pest species.

KEY WORDS Cerambycidae, Asian longhorned beetle, linden borer, symbiosis, gut microbiota

Microorganisms that inhabit insect guts can play important roles in the host's nutrition, development, resistance to pathogens, and reproduction (Brand et al. 1975, Brune 2003, Moran et al. 2005). Loss of microorganisms often results in abnormal development and reduced survival of the insect host (Eutick et al. 1978, Fukatsu and Hosokawa 2002). Some specific roles of microbiota in insect guts include lignocellulose digestion, methanogenesis and acetogenesis from H_2 and CO_2, nitrogen fixation, recycling uric acid nitrogen, maintenance of a low redox potential, and prevention of entry of foreign bacteria (Veivers et al. 1982, Ohkuma and Kudo 1996). Digestive symbioses appear most common among insects that feed on wood or other highly lignified plant materials. For example, associations with intestinal microbiota enable termites to be exclusively xylophagous (Breznak 1982, Breznak and Brune 1994).

Although microbes play important roles in the few systems where they have been studied, symbiotic associations remain unexplored for most insects, including many of the largest and most economically important families. Even baseline information on bacterial species composition is lacking, and such information is a prerequisite to subsequent studies on functionality, community processes, characterization of the net benefits to various members of the association, and opportunities for novel pest management strategies.

The family Cerambycidae consists of herbivorous endophytic insects that breed mostly in the woody tissues of trees. These insects play important roles in ecological processes such as nutrient cycling, biodiversity, and competition with economically damaging bark beetles (Schenk and Benjamin 1969, Miller 1985, Hanks et al. 1999, Dodds et al. 2001, Vance et al. 2003). Several species can also be serious economic pests of landscape and forest trees (Echaves et al. 1998, Hanks 1999, Hanks et al. 1999, Morewood et al. 2002, 2003, Fierke et al. 2005). Cerambycids are particularly noteworthy because their larvae develop deep within sapwood, a tissue characterized by very low nitrogen content and high lignification (Mattson 1980, Scriber and Slansky 1981, Haack and Slansky 1987). The cryptic subcortical nature of wood- and phloem-boring insects and the typically slow development associated with such a nutrient-poor substrate together contribute to another feature of this family—their high likelihood of being inadvertently transported into new geographic regions (Hoebeke 1994, 1999, Haack 2001). Cerambycids have thus been responsible for numerous damaging biological invasions. Some recent examples of damage to natural environments and human economies include the establishment of the Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) in the United States, the brown spruce longhorned beetle in Canada, agricultural and landscape pests such as the citrus borer and *Eucalyptus* borer in a variety of regions, and introduction of a *Monochamus*-vectored phytopathogenic nematode and subse-

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Table 1. Characteristics of 16S rRNA gene libraries constructed using gut bacterial communities of *A. glabripennis* and *S. vestita*

Insect/sampling location	Library (guts per library)	Clones sampled	Number of OTUs observed[a]			
			Ident.	3%	5%	10%
A. glabripennis						
WuJi, Hebei, China	hw4 (4)	44	37	9	8	6
Langfang, Hebei, China	hl9 (1)	15	13	7	6	6
	hl10 (1)	14	13	10	9	9
	hl11 (1)	4	4	4	4	4
	hl12 (1)	23	23	12	10	8
	Total (8)	100	87	36	29	21
S. vestita						
Oak Creek, WI	sv19 (2)	21	21	6	3	1
	sv21 (2)	19	17	5	3	2
Waukesha, WI	sv52 (1)	6	4	1	1	1
	sv53 (1)	21	18	3	3	1
	sv55 (1)	13	10	3	2	2
	Total (7)	80	69	14	8	3
Across all libraries		180	156	46	34	22

[a] OTU is defined as a group of sequences that are either identical or differ by no more than the indicated value.

quent vectoring by native beetles in Japan (Togashi 1988, Hanks 1999, Hanks et al. 1999, Nowak et al. 2001, Morewood et al. 2004).

The Asian longhorned beetle is native to eastern Asia and has become an important invasive pest in North America after its accidental introduction through wood packing material. It has become particularly problematic in urban and suburban areas of the Mid-Atlantic and Midwestern states. This pest is reported to have a broad host range in its native region, including maples, horsechestnuts, poplars, willows, elms, mulberries, and black locusts (Nowak et al. 2001, Morewood et al. 2002). In North America, it seems largely associated with maples. Because larvae bore deep into the wood, they are difficult to kill with biological or chemical pesticides. Hence, removal of infested and neighboring trees is currently the only practical control tactic. The linden borer, *Saperda vestita* Say, is a native species that primarily attacks *Tilia* sp. (i.e., linden and basswood), but occasionally attacks poplars (Drooz 1985). This beetle can be of considerable economic importance in the urban and suburban sectors where it can kill valuable ornamental and shade trees, and is likewise an important economic problem in nurseries. As with the Asian longhorned beetle, control options are limited.

Despite the ecological and economic importance of cerambycids, few studies have been conducted on their gut microbiota, and these have been limited to culture-dependent techniques and microscopic observations (Steinhaus 1941, Cazemier et al. 1997). However, many bacterial species are not amenable to culturing or distinguishable by microscopy and thus molecular methods commonly detect greater taxonomic diversity. Our goal was to explore the gut microsymbionts of two unstudied cerambycids, including one each of exotic and native origin. Our intention is to create a baseline for informing future studies on functionality, which in turn may suggest useful and environmentally benign management tools.

Materials and Methods

Insect Specimens. *A. glabripennis* larvae were collected in Wuji and Langfang, Hebei, China, on willow trees and preserved immediately in 100% ethanol. Willow trees in these sites are predominantly *Salix matsudana* with a few *Salix babylonica. S. vestita* larvae were collected from linden trees in Oak Creek and Waukesha, WI. Insects were surface sterilized by submersion in 70% ethanol for 1 min and rinsed in sterile water prior dissection. Insects were dissected in 10 mM sterilized phosphate-buffered saline (PBS) using dissection scissors and the guts were withdrawn with fine-tipped forceps, washed in PBS, and transferred to 1.5-ml microcentrifuge tubes with 500 μl of PBS.

Insect guts were sonicated (Branson Ultrasonics, Danbury, CT) in pools as indicated in Table 1 for 30 s and squeezed with a plastic pestle and vortexed at medium speed for 10 s to separate bacterial cells from the gut wall. The tubes were then centrifuged at low speed to pellet the insect tissues and undigested food. The supernatants were filtered using a 40-μm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) and filtered on a 12-μm Poretics, Polycarbonate filter (GE Osmonics, Trevose, PA). Samples were maintained at 4°C until DNA extraction.

DNA Extraction and Cloning. Microbial DNA was extracted from the extracted cells using the FastDNA SPIN kit for soil (Qbiogene, Carlsbad, CA) according to the manufacturer's directions, which involves mechanical lysis by bead beating followed by purification of DNA on a silica matrix. DNA from cells isolated from samples hw4, sv19, and sv21 was obtained using the method described by Broderick et al. (2004), which uses chemical detergents and enzymatic digestion to lyse the cells. The DNA was purified using chloroform and phenol extractions followed by isopropanol precipitation and Sepharose CL2B column purification (ICN Biomedicals, Aurora, OH). 16S rRNA genes were amplified with universal bacterial primers 27F and 1492R (Broderick et al. 2004). Purified polymerase chain reaction (PCR) products were

cloned into the pGEM-T vector (Promega, Madison, WI) according to the manufacturer's directions. Clones containing full-length inserts were identified by PCR amplifying the inserts from broth cultures grown from single colonies using the M13 vector primers. Full-length PCR fragments (≈1.5 kb) were purified and sequenced using primers SP6, T7, 27F, or 787R and a BigDye reaction mix (Perkin-Elmer, Wellesley, MA).

Sequence Analysis. We manually aligned all sequences using an ARB database (Ludwig et al. 2004). Each aligned sequence was added to an optimized maximum parsimony phylogenetic tree associated with the database using the maximum parsimony software incorporated into ARB. Phylum-specific masks were used to optimize the phylogenetic placement of each sequence (Lane 1991). We determined the taxonomic description of each sequence by identifying the group with which each sequence clustered. Sequences were assigned to operational taxonomic units (OTUs) by constructing a Jukes-Cantor corrected distance matrix in ARB (Ludwig et al. 2004), which was analyzed in the distance-based OTU and richness determination software package (DOTUR; Schloss and Handelsman 2005). Putative chimeras were identified by using CHECK CHIMERA (Cole et al. 2005), helical base pairing, and partial treeing (Huber et al. 2004).

Nucleotide Sequences. The sequences analyzed in this study have been deposited in GenBank under the accession numbers DQ279540-DQ279719.

Results

***A. glabripennis* 16S rRNA Gene Sequence Analysis.** We obtained 100 16S rRNA gene sequences from five different clone libraries using insects collected in WuJi and Langfang, Hebei, China. Analysis using DOTUR showed that when an OTU was defined as a group of sequences with no differences or 3, 5, or 10% difference, the composite sequence collection contained 87, 36, 29, and 21 OTUs (Table 1). Using the Chao1 nonparametric richness estimator (Chao 1984), we predicted that a complete census of the five libraries would yield >65 OTUs when defining an OTU as a group of sequences with no <5% difference from each other. Interestingly, using the Chao1 estimator, a complete census of the composite library from insects collected in WuJi was predicted to have found only 10 OTUs and the four libraries from Langfang were predicted to contain >65 OTUs.

Phylogenetic analysis of the 16S rRNA gene sequences obtained from the guts of *A. glabripennis* (number of sequences, $n = 100$) indicated that the commensal microbial community contained a broad taxonomic diversity (Table 2). Representatives of the Proteobacteria ($n = 45$), Bacteroidetes ($n = 3$), Actinobacteria ($n = 4$), and Firmicutes ($n = 48$) were identified. The most frequently identified type of 16S rRNA sequence clustered within representatives of *Melissococcus* spp. ($n = 21$), relatives of *Escherichia* spp. ($n = 11$), and *Paracoccus* spp. ($n = 10$). The sequence collections from insects collected in WuJi and Langfang overlapped in their membership, although the WuJi library lacked any representatives of the β-Proteobacteria, α-Proteobacteria, and the Bacteroidetes. Because of the relatively low sequencing coverage of the *A. glabripennis* clone libraries, additional sequencing will probably yield additional taxonomic diversity.

Table 2. Composition of gut bacterial communities of *A. glabripennis* and *S. vestita*

Phylogenetic affiliation of 16S rRNA sequences	Number of sequences found in each phylogenetic group	
	A. glabripennis ($n = 100$)	*S. vestita* ($n = 80$)
γ-Proteobacteria		
Escherichia spp.	11	42
Pseudomonas spp.	3	2
Xanthomonas spp.	2	0
Yersinia spp.	2	34
Uncultured Enterobacteriaceae spp.	0	2
β-Proteobacteria		
Comamonas spp.	2	0
α-Proteobacteria		
Acetobacter spp.	1	0
Paracoccus spp.	10	0
Rhodobacter spp.	7	0
Roseobacter spp.	1	0
Uncultured Rhizobiales spp.	2	0
Uncultured α-Proteobacteria	4	0
Bacterioidetes		
Flavobacterium spp.	1	0
Sphingobacterium spp.	1	0
Termite gut group spp.	1	0
Actinobacteria		
Actinomyces spp.	1	0
Aureobacterium spp.	1	0
Curtobacterium spp.	2	0
Firmicutes		
Lactococcus spp.	8	0
Leuconostoc spp.	9	0
Lactobacillus spp.	3	0
Melissococcus spp.	21	0
Enterococcus spp.	5	0
Bacillus spp.	2	0

Affiliation was assigned by determining which group each sequence clustered with in the ARB database phylogenetic tree.

***S. vestita* 16S rRNA Gene Sequence Analysis.** Five clone libraries constructed from *S. vestita* insects collected in Oak Creek and Waukesha, WI, were analyzed by sequencing 80 clones (Table 1). When an OTU was defined as a group of sequences with no differences, 3, 5, or 10% difference, the composite sequence collection contained 69, 14, 8, and 3 OTUs, respectively (Table 1). We predicted, using the Chao1 richness estimator, that a complete census of these clone libraries would find 14 OTUs when an OTU was defined as a group of sequences with no <3% difference from each other, a common definition of species (Hughes et al. 2001). Each of the three libraries constructed using single insects collected in Waukesha together accounted for an estimated five of these OTUs; however, when analyzed using a collector's curve, it was evident that the addition of each library to the analysis resulted in an increase in the number

of OTUs, indicating that there was insufficient sampling to obtain a reliable estimate of richness. Although the overall richness within a site increased with additional insect guts at the 3% OTU definition, the richness remained constant for an OTU definition of 10% difference.

In contrast to the taxonomic diversity found in *A. glabripennis*, phylogenetic analysis of the *S. vestita*--associated community showed that most members were representatives of the γ-Proteobacteria ($n = 80$, Table 2). Among the γ-Proteobacteria, the most frequently identified type of 16S rRNA sequence clustered with relatives of *Escherichia* spp. ($n = 42$) and *Yersinia* spp. ($n = 34$). The most substantive difference between the commensal bacterial communities of *A. glabripennis* and *S. vestita* is the difference in taxonomic diversity. Although this tabulation could rise with increased sampling, it is noteworthy that all of the sequences found in *S. vestita* were also found in *A. glabripennis*, with the exception of the uncultured Enterobacteriaceae sequences ($n = 2$; Table 2).

Discussion

Based on 16S rRNA sequencing analysis, *A. glabripennis* and *S. vestita* showed substantial differences in taxonomic diversity of their gut symbionts. 16S rRNA genes that affiliate with the Enterobacteraceae of the γ-Proteobacteria were found in 78 of the 80 sequences sampled from the *S. vestita* microbial communities, whereas representatives of the γ-Proteobacteria, β-Proteobacteria, α-Proteobacteria, Bacteriodetes, Actinobacteria, and Firmicutes were found in the *A. glabripennis* microbial communities. Among the γ-Proteobacteria sequences, there was considerable overlap between the two insects, suggesting that the taxa found in *S. vestita* were a subset of those found in *A. glabripennis*. Members of the Enterobacteraceae in the γ-Proteobacteria are commonly found in commensal gut communities from humans to insects and often aid in vitamin biosynthesis, pheromone production, and degradation of plant compounds (Breznak 1982, Xu and Gordon 2003). More intensive sequencing and broader geographic and host tree sampling will help determine the biological significance of this result.

The gut microbial community of *S. vestita* was recently studied by culturing, which yielded two strains of *Sphingobium yanoikuyae*, an α-Proteobacterium, in all larvae surveyed (Delalibera et al. 2005). However, 16S rRNA sequences from this species were not detected in the *S. vestita* and *A. glabripennis* culture-independent 16S rRNA sequence collections. This is likely caused by the low relative abundance of *S. yanoikuyae* in the *S. vestita* gut and the limited number of clones sequenced. The 16S rRNA genes of seven of eight additional isolates from the *S. vestita* culturing study were most similar to 16S rRNA gene sequences of the Enterobacteraceae found here by culture-independent means. An additional isolate similar to isolates of the β-Proteobacterium, *Acidovorax* sp., were found in the *S. vestita* culture collection but not in the 16S rRNA clone library.

The two wood-boring insects studied in this investigation are largely recalcitrant to biological and chemical pesticides because of their protected microhabitat and the practical difficulties of treating large trees. The description of the microbial membership of the gut community is the first step in understanding structure–function relationships within commensal microbial communities of these insects for purposes of pest management, biodiversity exploration, and understanding the roles of symbioses in colonizing physicochemically severe microhabitats.

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